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Report sent to NASA concerning the USU grant

The goals of the study were to explore the effects of microgravity upon peroxidases in super dwarf wheat. The study was to explore peroxidase activities and isozyme patterns associated with different plant organs and to determine whether any changes in peroxidases in microgravity were related to altered lignin deposition or to hydrogen peroxide formation in the plant tissues.

Conditions were established for successful growth of the plants in a containerized environment using seeds that were just germinated. Seeds had been surface sterilized and heat treated in an attempt to limit growth of associated microbial contaminants. The superdwarf wheat plants were flown for 8 days in the middeck space on Discovery. Both flight and ground control plants were diseased showing growth of a tan mycelium from exposed roots and leaf surfaces. The 1995 research at Utah State University in the research group of Dr. Anne Anderson has concentrated upon understanding the nature of the microbial infection and the effects of the microbe upon plant peroxidase activity.

The infection was determined to arise from an endophytic fungus that was seed borne. The fungus resembles an *Acremonium* species in its mycelial growth form and in the generation of hyaline spores on solid medium. The same fungus was identified by our laboratory in the super dwarf wheat seed used by Salisbury et al. in the MIR mission.

The fungus is asymptomatic upon growth of the seeds in open culture under high light. The symptoms observed from the space-flown plants were reproduced by inoculation of heat-cleaned seed with spores of the purified, cultured fungus and growth in enclosed containers. Infection of clean plants from a single inoculated plant was observed under containerized growth.

Infection of the plants with the *Acremonium* fungus caused alterations in the peroxidase isozymes associated with the wheat cell walls. The changes in the apoplastic peroxidases differed between the root, shoot and the crown regions of the plant. Certain of the isozymes detected in the fungally - colonized plants were identified as those that were also found in the tissues from the plants grown under microgravity. Thus the microgravity study involved not only effects of gravity on the plant peroxidases but also effects upon the plant -microbe association .

Task progress.

Anderson and Bishop have found that the super dwarf wheat seed distributed by Dr. Bruce Bugbee for NASA-related research has endophytic fungi that are seed-borne. Several seed lots have been examined and the infected seed include that flown on Discovery in February 1995 for the Hood and Anderson grant as well as the seed used for the December 1995 MIR mission flown by Salisbury et al. Two fungal species have been identified consistently in the super dwarf seed after surface sterilization. Fungal cultures are identified as *Acremonium* and *Cladosporium* species. The extent of infestation of the seed is variable from 25 % to over 90 %.

The superdwarf wheat seed has been freed of the endophytes by prolonged heat treatment at 50°C. The germination of the seed is not impaired by the process. Plants raised under greenhouse conditions from this disinfested seed yield seed that has no endophyte contamination.

Inoculation of disinfested seed by the purified *Acremonium* culture and growth of the plants under containerized conditions resulted in the duplication of the symptoms observed in the plants flown for 8 days in the chambers housed in the middeck of Discovery. The plants show chlorosis and emergence of tan mycelia from the leaves and exposed root tissues.

The methods for analysis of peroxidase activities have been developed. Activity is determined by spectrophotometric methods on apoplastic preparations as well as in cell homogenates. Activities for enzymes that are cytoplasmic have been performed to test the rigor of the apoplastic extraction. Isoelectric focussing has been used to separate the peroxidase isozymes. Two substrates have been used in staining these gels to examine substrate specificity for the individual isozymes. These techniques show that the presence of the *Acremonium* fungus alters isozyme patterns and activity levels in plants that were grown from disinfested seed that was inoculated with fungal spores. The responses differ in between the root, crown and leaf tissues. We find that the fungus induces the same bands of peroxidase that were detected in pooled extracts from space grown plants. Thus from this study it is not possible to elucidate the effects of microgravity from that of the fungal infection. A similar conclusion is made with respect to the evaluation of lignin deposition and hydrogen peroxide production. The methods developed to test these parameters yield reproducible results with sterile grown wheat. Changes were observed in the microgravity plants but we have not yet determined whether the alterations are due to the presence of the fungus rather than a response to zero gravity.

In an attempt to understand the responses of the plant at the molecular level we have initiated cloning of the genes that encode the apoplastic peroxidases in superdwarf wheat. We have PCR products that possess sequences that have high similarity with sequences in other plant peroxidase genes. We are determining whether these sequences are assigned to genes that are upregulated upon fungal challenge.

Earth benefits

The studies on superdwarf wheat illustrate that growth conditions play a major role in determining plant vigor. Growth of wheat seeds harboring an endophytic fungus under suboptimal conditions resulted in the fungus becoming pathogenic. These findings indicate the problems of latent pathogens in our food sources. The findings also demonstrate how little is known at the molecular level about the influence of stress on whether the plant -microbe interaction is benign or has the potential to reduce plant growth. We need to understand how plants interact with a diversity of microbes to maximize food production in our world of increasing human population. Knowledege of the processes that limit pathogen development may promote our abilities to boost crop production without the use of chemicals that have harmful environmental impact. Thus the NASA funded studies will help in the understanding of how to attain sustainable agriculture on earth.

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The work discussed in this report hhas been published in Phytopathology:

D.L. Bishop, H. G. Levine, B.R. Kropp and Anne J. Anderson. Seedborne Fungal Conatmination: Consequences in Space-Grown Wheat. Phytopathology 87:1125-1133 (1997).

A copy of the paper is attached.

Seedborne Fungal Contamination: Consequences in Space-Grown Wheat

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ABSTRACT

Bishop, D. L., Levine, H. G., Kropp, B. R., and Anderson, A. J. 1997. Seedborne fungal contamination: Consequences in space-grown wheat. Phytopathology 87:1125-1133.

Plants grown in microgravity are subject to many environmental stresses that may promote microbial growth and result in disease symptoms. Wheat (cv. Super Dwarf) recovered from an 8-day mission aboard a NASA (National Aeronautics and Space Administration) space shuttle showed disease symptoms, including girdling of leaf sheaths and chlorosis and necrosis of leaf and root tissues. A *Neotyphodium* species was isolated from the seed and leaf sheaths of symptomatic wheat used in the spaceflight mission. Certain isozymes of a peroxidase unique to extracts from the microgravity-grown plants were observed in extracts from earth-grown *Neotyphodium*-infected plants but were not present in noninfected wheat. The endophytic fungus was eliminated from the wheat seed by prolonged heat treatment at 50°C followed by washes with water at 50°C. Plants from wheat seed infected with the *Neotyphodium* endophyte were symptomless when grown under greenhouse conditions, whereas symptoms appeared after only 4 days of growth in closed containers. Disease spread from an infected plant to noninfected plants in closed containers. Dispersion via spores was found on asymptomatic plants at distances of 7 to 18 cm from infected plants. The size and shape of the conidia, mycelia, and phialide-bearing structures and the ability to grow rapidly on carbohydrates, especially xylose, resembled the characteristics of *N. chilense*, which is pathogenic on orchard grass, *Dactylis glomerata*. The *Neotyphodium* wheat isolate caused disease symptoms on other cereals (wheat cv. Malcolm, orchard grass, barley, and maize) grown in closed containers.

Additional keywords: fungal endophyte.

Plant-microbe interactions rarely have been considered significant components of the plant growth experiments thus far conducted in space. Although it has been possible to maintain asepsis for short-term plant growth studies in microgravity (19,20), this approach is unrealistic for longer flights, when environmental recycling and food production will be required (5). The spaceflight environment is not sterile, and microbes that can colonize plants have been isolated from spaceflight hardware (4,26,30,31). The few investigations available report a general pattern of increased growth rates for microbial organisms exposed to microgravity (10, 14,33).

Plant growth in space may provide a greater opportunity for potential pathogens to colonize plants and, thus, facilitate pathogenesis. Under the controlled environments in spaceflight experiments, plants are subjected to environmental stresses, including elevated carbon dioxide (CO_2), low photosynthetic photon flux density, and high relative humidity (RH). Plant growth and metabolism are altered in microgravity, e.g., key enzymes associated with lignification change, less vigorous and abnormal seed is produced, and photosynthetic rate and light-saturated electron transport is decreased (7,12,32). These conditions and changes in plant physiology may affect host defenses and make plants more susceptible to infection by pathogens and organisms that normally do not cause disease. The work in this paper originated as a result of the CHROMEX-06 spaceflight experiment conducted aboard the U.S. space shuttle Discovery during the STS-63 mission. The original goal was to investigate the effect of microgravity on the activity and composition of peroxidases in wheat (*Triticum aestivum* L. 'Super Dwarf'). Although the seeds underwent a rigorous seed surface-sterilization, nearly one-half of the seedlings returned from the mission with visual fungal contamination. We report on studies conducted to understand the source of contamination and the damage to wheat caused by the fungi. Our findings are the first to document colonization of wheat by a *Neotyphodium* species.

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MATERIALS AND METHODS

Plant growth in spaceflight environments. Seventy-two wheat (cv. Super Dwarf) plants were grown in a plant growth unit (PGU) in a middeck locker of the U.S. space shuttle Discovery during an 8-day mission. The PGU housed six individual, enclosed plant growth chambers (PGCs). Prior to planting, seed was surface-sterilized by submersion in 70% ethanol for 1 min and 25% sodium hypochlorite containing 0.2% Triton X-100 with vigorous shaking at 200 rpm for 20 min. Seed was rinsed five times with sterile deionized water (22°C) and transferred to sterile petri dishes containing 22 ml of 2.0% water agar supplemented with 0.5% sucrose. Plated seeds were kept at 4°C for 4 days to synchronize germination. Twelve 36-h-old germinated seedlings were planted (24 h prior to lift-off) in individual Nitex (50-μ mesh nylon; Teckco Inc., Lancaster, NY) planting pockets fitted into the foam base in each PGC. Foam root barriers were placed in each planting pocket on both sides of each seedling.

Environmental parameters used for plant growth are described by Krikorian and Levine (16) and Levine and Krikorian (18). Each foam base was supplied with 200 ml of sterile one-half strength Schenk and Hildebrandt (SH) nutrient medium (27) supplemented with 0.5% sucrose prior to planting. Plants were grown under a 24-h photoperiod, with light intensities ranging from 30 to 60 μmol m^{-2} s^{-1}, 90 to 100% RH, and carbon dioxide that increased from ambient to 150,000 ppm by the end of the mission. Ground-control plants were grown under the same environmental conditions as the flight

plants (except for microgravity and other spaceflight-associated factors, such as increased levels of radiation plus launch and landing vibration loads) supplied by an orbital environmental simulation chamber at the John F. Kennedy Space Center, Cape Canaveral, FL. Sections of leaf sheath and meristematic tip regions were cultured on potato dextrose agar (PDA; Difco Laboratories, Detroit) from the space-flown plants at the end of the mission to culture microbial contaminants.

Wheat seed sources. Spring wheat (*T. aestivum*) cv. Super Dwarf initially was obtained from CIMMYT, Mexico City, by B. Bugbee, Plant Soils and Biometerology Department, Utah State University, Logan, who grew additional accessions (lots 1, 2, and 4). Seed lot 1 was grown in 1991 under greenhouse conditions. Seed lot 2, used in both U.S. space shuttle (STS-63) and U.S. NASA (National Aeronautics and Space Administration)/Russian Mir wheat experiments in 1995, was derived by multiplication of the 1991 seed under greenhouse conditions in 1993. Seed lot 3 was obtained from seed lot 2 by growth in a mixture of 1:1 peat/vermiculite under greenhouse conditions, with high light intensity (1,000 μmol m^{-2} s^{-1}), day and night temperatures of 22 and 17°C respectively, 50% RH, and adequate nutrients. Seed lot 4 was distributed to the NASA Centers for use in plant space biological research. Endophyte-free seed lot 5 was obtained by a 50°C heat treatment, as described below, and increased in sterile environmental-control chambers. A newly released wheat seed cultivar, USU-Apogee (lot 6), which was bred specifically for space-related research (B. Bugbee, Utah State University), also was examined for endophytic contamination.

Identification of endophytes. Seed was surface-sterilized, as described above, with ethanol and sodium hypochlorite treatments. Fungi isolated from the seed after growth on PDA plates for 2 weeks were purified by dilution plating to obtain single spores. Conidia were suspended in 5 ml of sterile double-deionized water and filtered through siliconized glass wool to remove any mycelia prior to storage or use. Fungi were cultured also from sections of diseased wheat leaves that returned from the space mission. The fungal isolates were stored at −80°C in 15% glycerol, either as a plug of mycelium from a PDA plate or as a pure spore suspension. Cultures were maintained by transfer of inoculum plugs, ≈1 cm in diameter, to fresh PDA plates every 14 days.

Morphological characteristics of spores, mycelia, and plate-grown colonies (23,24,34,36) were used in the identification of the *Neotyphodium* isolate recovered from microgravity-exposed wheat leaves. The isolate was compared to *N. coenophialum* isolated from seed of endophytic *Festuca arundinacea* (cv. Kentucky 31), as described above. A 1% aqueous, aniline blue, lactic acid stain (2) was used to stain hyphae and spores. Photographs were obtained under 100× magnification. Colony diameters (millimeters) and the coloration of the mycelium on the reverse side of growth plates were examined on 1.8% water agar and Murashige and Skoog medium (25) amended with 4% filter-sterilized fructose, glucose, mannose, sucrose, or xylose to further characterize the *Neotyphodium* species (24).

Immunoblot analysis with polyclonal antibodies raised against *N. coenophialum* (provided by C. West, University of Arkansas, Fayetteville, in collaboration with T. Jones, USDA Forage and Range Facility, Logan, UT) was performed with proteins extracted from the *Neotyphodium* isolates from fescue and wheat grown for 2 weeks on PDA plates or from germinated seeds of endophyte-positive wheat and fescue. Proteins extracted from 2-week-old mycelia of *Fusarium culmorum* (strain 409), a wheat pathogen (17,28), and from germinated aseptic Super Dwarf seeds served as controls. Proteins were extracted by grinding 0.2 g of mycelial mass in liquid nitrogen prior to transfer to 0.2 ml of 50 mM phosphate buffer, pH 6.0. The extracts were boiled for 2 min in sodium dodecyl sulfate (SDS) buffer (13), and 20 μg of protein of each sample was separated on a 10% polyacrylamide gel by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to an Immobilon membrane (Boehringer Mannheim, Indianapolis, IN) with a Bio-Rad (Hercules, CA) electrophoretic transfer

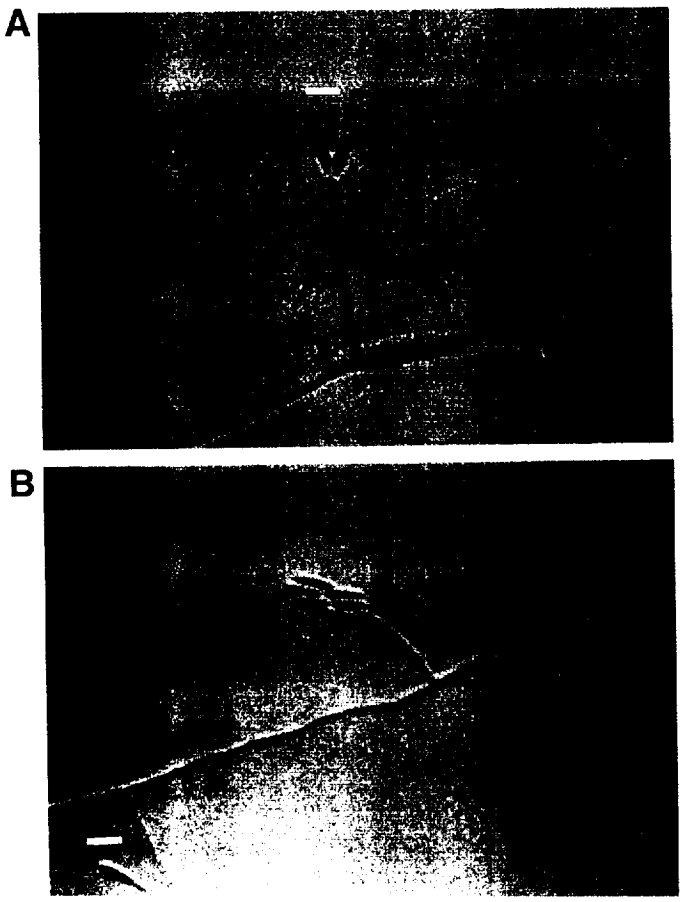

Fig. 1. Morphology of fungal mycelia, phialide structures, and conidiospores from the *Neotyphodium* isolate from **A,** wheat cv. Super Dwarf, compared to those from **B,** the fescue isolate, *N. coenophialum*. The preparations were from 14-day-old potato dextrose agar plates and were stained with an aniline blue, lactic acid stain, as described in text. The bar represents 10 μm.

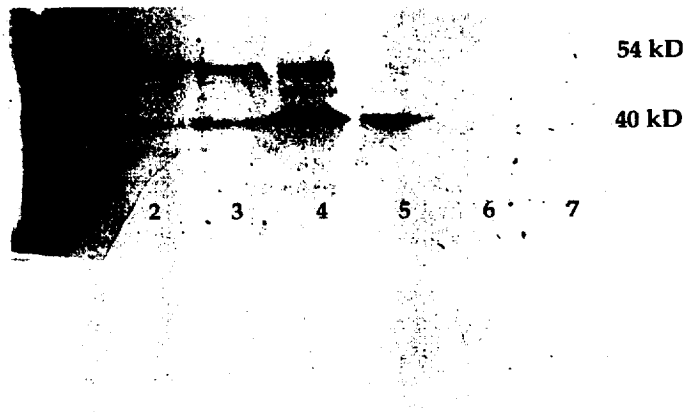

Fig. 2. Immunodetection of protein from wheat and fescue *Neotyphodium* isolates, with antibody generated to *N. coenophialum*. *Neotyphodium*-specific polyclonal antibodies were reacted with extracts from lane 1, *Neotyphodium* mycelia isolated directly from surface-sterilized endophytic *Festuca arundinacea*, or lane 2, wheat cv. Super Dwarf seeds (lot 2) after germination on potato dextrose agar (PDA); lane 3, mycelia of *N. coenophialum* after growth on PDA for 2 weeks; lane 4, mycelia of the *Neotyphodium* isolate from wheat after growth on PDA for 2 weeks; lane 5, mycelia of the *Neotyphodium* isolate from *Achaetherum robustrum* after growth on PDA for 2 weeks; lane 6, negative control, heat-treated, endophyte-free 14-day-old cv. Super Dwarf germinated on PDA; and lane 7, *Fusarium culmorum* mycelia after growth on PDA for 2 weeks. Procedures were as described in text.

apparatus set at 8 mA for 1 h. An identical gel with an additional protein standard to determine molecular weight was stained for total protein with Coomassie blue (13). The nitrocellulose membrane was allowed to react with the antibodies to *Neotyphodium* before treatment with a protein A-alkaline phosphatase conjugate (Sigma Chemical Co., St. Louis) and detection of alkaline phosphatase activity (11).

Generation of endophyte-free seed. Seed was submersed in sterile double-deionized water containing 0.2% Triton X-100 and incubated with vigorous shaking at 150 rpm for 40 min at temperatures of either 22, 40, or 50°C. This procedure was done twice. After incubation, the seed was washed three times with sterile double-distilled water equilibrated to the incubation temperature. The seed was surface-sterilized by immersion in 25% sodium hypochlorite containing 0.2% Triton X-100, washed five times with sterile water, transferred to plates of PDA, and incubated at 22°C. The type of endophytic contamination, percent seed sterility, and germination were recorded for each of the treatments.

Seed surface-sterilization treatments. Seeds were immersed in concentrated (96.4%) sulfuric acid (H_2SO_4) for 5 min or 30% hydrogen peroxide (H_2O_2) for 10 min, followed by extensive washing in sterile distilled water. Seeds were transferred to plates of PDA, kept at 4°C for 4 days to synchronize germination, and kept at 22°C under incandescent lighting to assess percent sterility and germination over an 8-day period.

Establishment of symptoms on wheat and other hosts. Symptoms caused by inoculation of wheat cv. Super Dwarf with the *Neotyphodium* isolate from wheat were compared to symptoms on microgravity-grown wheat. Endophyte-free seed (lot 5) was surface-sterilized with 25% sodium hypochlorite in 0.2% Triton X-100 with shaking for 20 min at 200 rpm. After five rinses with sterile distilled water, seed was immersed in a suspension of *Neotyphodium* spores (10^6 spores per ml) in 1% carboxy-methylcellulose for 2 min, which was obtained as described above from a 2-week-old *Neotyphodium* culture on PDA. Five seeds were transferred to each Magenta box (Magenta Corporation, Chicago) containing sterilized vermiculite (200 ml) and sterile water (100 ml). Each treatment consisted of 100 seeds, and each treatment was replicated five times. The closed boxes were placed at 26°C under a 24-h photoperiod provided by incandescent lighting (110 μmol m^{-2} s^{-1}) for 8 days. As a control, seed was treated with sterile water instead of the spore suspension.

This procedure was repeated with other cereals—fescue, orchard grass, wheat cv. Malcom, barley cv. Steptoe, and maize cv. Spancross—to examine the potential of the *Neotyphodium* isolate from wheat to cause disease on other plant species. Symptoms on each inoculated host plant were recorded at 8 and 16 days for five separate trials. A dose-response relationship was examined by inoculating the seed with suspensions of 10^2, 10^3, 10^4, 10^5, 10^6, and 10^7 spores per ml. Emergence was recorded at 8 days and symptoms at 16 days. The presence of *Neotyphodium* was confirmed by transferring sections of the diseased wheat stems to plates of PDA and observing the characteristic coloration of the mycelium and the size and shape of the spores and phialides. These studies were performed twice, with two replicates each.

The potential for fungal dispersion from *Neotyphodium*-infected Super Dwarf seed was evaluated by planting a single, surface-sterilized *Neotyphodium*-infested seed from lot 2 in a Nitex planting pocket in each of six PGCs. The other 11 pockets were planted with surface-sterilized, heat-treated, endophyte-free seeds. The plants were grown under conditions similar to those used in spaceflight: high RH (greater than 90%), a stable temperature of 28°C, and low incandescent lighting during a 24-h photoperiod. Plants were watered and fertilized with one-half strength SH nutrient medium (27). Observations of microbial spread and symptom development on neighboring plants were recorded daily, and plants were inspected microscopically at 8 days for five separate trials.

Endophyte-infected wheat seed (lot 2) was planted under open greenhouse conditions to produce asymptomatic plants or in closed containers to permit symptom development. Sections of leaves, sheaths, and roots stained with 1% aqueous aniline blue, lactic acid stain (2) were examined microscopically at 100× magnification

TABLE 1. Colony diameters of *Neotyphodium* isolates from *Festuca arundinacea* and *Triticum aestivum* on agar amended with 4% fructose, glucose, mannose, sucrose, or xylose

Carbohydrate[y]	Colony diameter (mm)[z]	
	Neotyphodium wheat isolate	*N. coenophialum*
Fructose	60 ± 5 a	6.4 ± 2 b
Glucose	48 ± 3 b	0.8 ± 2 c
Mannose	45 ± 5 b	11 ± 4 a
Sucrose	32 ± 2 c	9.3 ± 3 a
Xylose	46 ± 5 b	0.3 ± 2 c

[y] Media were inoculated with a plug (1 cm diameter) from 8-day-old potato dextrose agar cultures of *Neotyphodium* isolates.

[z] Plates were incubated at 26°C, and colony diameters were measured at day 30. Data were transformed by a log transformation and subjected to two-way factorial analysis. Data are represented as means with the standard error of five replicated samples for each of two trials. Colony diameter on various carbohydrate-containing media was significantly greater for the *Neotyphodium* wheat isolate compared to the *Neotyphodium* fescue isolate (α = 0.05). Means in the same column followed by the same letter are not significantly different.

TABLE 2. Effect of heat treatment and washing on elimination of microbes from wheat seed

Seed lot (year)	Temperature treatment (°C)[y]	Water wash (°C)[y]	Seeds without contamination (%)[z]	Germination (%)[z]
1 (1991)	22	22	7 ± 4.0 d	52 ± 10.7 c
	40	40	17 ± 6.0 c	66 ± 5.3 b
	50	40	67 ± 16.0 b	74 ± 11.3 a,b
	50	50	100 ± 0.0 a	80 ± 5.0 a
2 (1993)	22	22	7 ± 3.3 d	55 ± 10.0 b
	40	40	20 ± 3.3 c	73 ± 4.0 a
	50	40	70 ± 13.3 b	74 ± 2.6 a
	50	50	100 ± 0.0 a	95 ± 6.7 a
3 (1993)	22	22	28 ± 6.0 d	78 ± 6.3 b
	40	40	42 ± 7.3 c	77 ± 4.0 b
	50	40	74 ± 9.3 b	81 ± 5.3 b
	50	50	100 ± 0.0 a	89 ± 5.3 a
4 (1995)	22	22	70 ± 13.3 c	87 ± 4.0 a,b
	40	40	80 ± 6.0 b,c	80 ± 13.3 b
	50	40	89 ± 5.3 b	84 ± 5.3 b
	50	50	99 ± 2.7 a	94 ± 5.3 a
5 (1995)	22	22	98 ± 4.0 a	86 ± 5.3 a
	40	40	99 ± 6.0 a	85 ± 3.3 a
	50	40	100 ± 0.0 a	88 ± 4.0 a
	50	50	100 ± 0.0 a	87 ± 14.6 a
6 (1996)	22	22	83 ± 5.0 c	80 ± 5.1 b
	40	40	86 ± 6.0 c	87 ± 3.0 a
	50	40	92 ± 0.0 b	89 ± 2.0 a
	50	50	100 ± 0.0 a	90 ± 3.6 a

[y] Seed was subjected twice to heat treatments at temperatures of 22, 40, or 50°C for 40 min each and to water washes equilibrated to the indicated temperatures. After the heat treatment protocol, seed was surface-sterilized with 70% ethanol for 1 min and 25% sodium hypochlorite for 20 min before washing five times with sterile double-deionized water.

[z] Seed was examined for the presence of endophytic contaminants, as described in text, and germination was assessed. Data were transformed with an arcsine square-root transformation and are represented as back-transformed means with a standard error of five replications, each with 100 seeds. Significant differences in percent seeds without contamination and germination between heat treatments were apparent in seed lots 1 through 4 and 6 (α = 0.05). Endophyte-free seed lot 5 showed no difference in percent seed without contamination and germination for these treatments. Means in the same column and seed lot followed by the same letter are not significantly different.

for the presence of fungal hyphae and spores. This experiment was repeated three times.

Statistical analyses. Data for percent sterility and germination were subjected to arcsine square-root transformation. Log transformation of colony diameters was analyzed by analysis of variance. Comparisons of means were made by Tukey's multiple comparison test at $\alpha = 0.05$.

Peroxidase composition. Peroxidase isozymic composition from microgravity- and earth-grown plants was compared to that from *Neotyphodium*-infected and noninfected plants. Leaf sheaths and roots were ground in 0.01 M K_2HPO_4, 0.2 M KCl, pH 6.0, buffer at 4°C, and cell debris was removed by high-speed centrifugation, 15,000 × *g* (SS34 rotor; Sorvall Instruments, Du Pont Co., Wilmington, DE) for 5 min at 4°C. Peroxidase isozyme composition in 1 µg of protein, as determined by a bicinchoninic acid assay (Pierce Chemical Co., Rockford, IL), was examined by isoelectric focusing (22). Ampholytes (Pharmacia Biotech, Uppsala, Sweden) were used at 1.0% in the pI range of 3 to 10. Gels were run at 4°C and 4 W of constant power for 1 h, increasing to 8 W for 2 h. Gels were stained for peroxidase activity in Tris buffer (pH 7.5), with 0.15% (wt/vol) hydrogen peroxide and catechol/*p*-phenyldiamine as the substrates (1,22). The isoelectric points of the proteins were determined by isoelectric point markers (FMC BioProducts, Rockland, ME).

RESULTS

Growth under spaceflight conditions. Approximately 50% of the initial 72 seedlings (lot 2) grown for 8 days in microgravity aboard the U.S. space shuttle Discovery showed signs of fungal contamination, including abundant white cottony aerial mycelium with profuse mycelial growth on elevated roots, leaf sheaths, and wheat culms. The leaves of infected plants were chlorotic, twisted, and necrotic at the tips. Similar symptoms were observed in about 30% of the control plants grown under identical conditions on the ground.

Identification of *Neotyphodium* from seed. Endophytic fungi were isolated from cv. Super Dwarf seed (lot 2) used in the spaceflight mission. Mycelia emerged from seed as it germinated on PDA plates. Two fungi were observed most frequently from lot 2 seed. One was identified as a *Cladosporium* species (34) by its spore characteristics and brown to black mycelium. This fungus is a known seedborne endophyte of wheat (28). The second fungus was identified as a *Neotyphodium* species, based on the size and morphology of conidia and conidiogenous cells and colony characteristics in vitro (8,23,24,34,36). The small hyaline conidia produced white to slightly pink or tan mycelia, with tan to brown coloration on the reverse side of the PDA plates after 2 weeks. The smooth, hyaline conidia were slightly oblong with an obtuse apex and were ≈10 to 20 µm long and 1 to 2 µm wide. The size and morphology of conidia and fungal mycelia from pure cultures of the *Neotyphodium* isolate from wheat are shown in Figure 1 compared to *N. coenophialum*, an endophyte of fescue. The conidia and spore-bearing phialides are characteristic of other *Neotyphodium* species (8,15,23,24,34,36), with a close resemblance to those of *N. chilense* (24) previously isolated from *Dactylis glomerata*.

Antibodies raised against *N. coenophialum* (Fig. 2) reacted with two proteins of 54 and 40 kDa in mycelial extracts prepared from isolates of *Neotyphodium* from wheat, fescue, and sleepy grass grown on PDA. Proteins extracted from *Fusarium culmorum* and noninoculated wheat cv. Super Dwarf did not react with the *Neotyphodium*-specific antibodies. Our results confirm the finding that the polyclonal antibodies raised against *Neotyphodium* recognize different *Neotyphodium* species.

The *Neotyphodium* isolate from wheat cv. Super Dwarf grew more rapidly on PDA and produced more conidia than *N. coenophialum* isolated from fescue (Table 1). Its ability to grow rapidly and form sectors on xylose- and glucose-containing media clearly distinguished the wheat isolate from *N. coenophialum*. These characteristics and the potential of the *Neotyphodium* isolate from wheat to cause disease are similar to traits of *N. chilense* from *D. glomerata* (24).

The fungus cultured from the leaf sheaths of space-flown plants had the same growth characteristics as the *Neotyphodium* isolate from wheat seed (lot 2). *Neotyphodium* also was isolated from

TABLE 3. Effect of surface-sterilization procedures on elimination of microbial endophytes from wheat cv. Super Dwarf seed

	Seed lot (year)[z]			
	4 (1995)		5 (1995)	
Treatment[y]	Without contamination (%)	Germination (%)	Without contamination (%)	Germination (%)
None	0 ± 0.0 c	85 ± 5.0 a	80 ± 5.3 c	79 ± 5.0 c
Concentrated H_2SO_4	0 ± 0.0 c	80 ± 10.3 a	99 ± 2.7 a,b	87 ± 5.3 b
30% H_2O_2	10 ± 20.0 c	82 ± 6.3 a	100 ± 0.0 a	95 ± 2.6 a
Sodium hypochlorite	70 ± 13.3 b	87 ± 4.0 a	98 ± 4.0 b	86 ± 5.3 b
Sodium hypochlorite and 50°C heat and wash	100 ± 0.0 a	87 ± 4.0 a	100 ± 0.0 a	93 ± 2.6 a

[y] Surface-sterilization and 50°C heat treatments were performed, and the extent of microbial contamination and germination of wheat seed were assessed on potato dextrose agar, as described in text.

[z] Data were transformed by arcsine square-root transformation and are represented as back-transformed means with standard error of five replications, each with 100 seeds. There was a significant seed lot-treatment interaction for percent sterility with the alternative sterilization protocols for seed lots 4 and 5 ($\alpha = 0.05$). Seed lots 4 and 5 also showed overall differences in percent symptoms and germination among the sterilization treatments ($P \leq 0.001$). Means in the same column followed by the same letter are not significantly different.

TABLE 4. Response of wheat cultivars to inoculation by *Neotyphodium* spores

		Inoculum dose (spores per ml)						
Cultivar[z]	Plant response	10^2	10^3	10^4	10^5	10^6	10^7	0
Malcom	Emerged	7 ± 2	4 ± 2	3 ± 1	1 ± 0	3 ± 2	3 ± 2	15
	Diseased	10 ± 3	12 ± 3	13 ± 2	14 ± 0	14 ± 1	15 ± 0	0
Super Dwarf	Emerged	11 ± 1	9 ± 2	12 ± 1	12 ± 3	9 ± 2	10 ± 1	15
	Diseased	5 ± 2	5 ± 2	8 ± 1	9 ± 2	10 ± 3	12 ± 3	0

[z] Cv. Malcom or Super Dwarf seed was inoculated with spores of *Neotyphodium* at concentrations of 10^2 to 10^7 spores per ml, as described in text. The plants were grown for 8 days, and emergence was scored. Disease was scored at 16 days. Data are the number of plants that emerged or were diseased from a total of 15 planted seeds. The study was repeated twice, and the mean with the standard error is provided.

seed lots 1, 3, and 4 of Super Dwarf when seed was subjected only to surface-sterilization protocols. In collaboration with W. Campbell at Utah State University, Logan, we demonstrated that the *Neotyphodium* endophyte also was present in seed lot 2 used in the NASA/Mir mission. Wheat cv. USU-Apogee also contained seedborne endophytes but to a lesser extent. Approximately 4% of Apogee seed was infected with the *Neotyphodium* endophyte. This degree of infection is less than the level observed with cv. Super Dwarf, the maternal parent of USU-Apogee.

Elimination of seedborne endophytes. Heat treatment of seed at 50°C in addition to surface-sterilization treatments consistently eliminated endophytes (Table 2). Heat treatments at 40°C followed by subsequent water washes equilibrated at 40°C reduced the amount of contamination by 10% compared to seed that received only a surface-sterilization treatment. Heat treatment at 50°C with a 40°C water wash further decreased fungal growth from the seed by ≈35%. Prolonged heat treatment at 50°C followed by 50°C equilibrated washes completely eliminated endophytic fungi from seed without reducing germination. There were significant differences in percent symptoms between heat treatments in seed lots 1 through 4 and 6 ($P \leq 0.001$) (Table 2).

To test the efficacy of our methods to generate endophyte-free seed, seed from lots 2 and 3 was subjected to the 50°C heat treatment and 50°C wash protocol. Seedlings were grown for 8 days in PGCs identical to those used in the CHROMEX-06 spaceflight experiment for three separate trials. There were no disease symptoms and no visible fungal growth on roots or leaves. Sections of root and leaf tissues from these plants were free of any microbial contamination when examined microscopically. The seed generated from lot 5 seed was endophyte-free (Tables 2 and 3) and produced disease-free seedlings.

Alternative surface-sterilization treatments involving immersion in concentrated sulfuric acid and 30% hydrogen peroxide for 10 min did not eliminate seedborne endophytes (Table 3). There was a significant seed lot-treatment interaction for percent sterility with the alternative sterilization protocols for seed lots 4 and 5 ($P \leq 0.001$). Seed lots 4 and 5 also showed overall differences in percent sterility and germination compared to the sterilization treatments (Table 3) ($P \leq 0.001$).

***Neotyphodium* and symptoms on infected wheat.** After surface-sterilization and growth in closed containers, seedlings from lots 2 and 4 displayed symptoms identical to those observed on the spaceflight mission. Similar symptoms also were observed in plants grown from lot 5 after inoculation with *Neotyphodium* spores and growth in closed containers. There was abundant mycelial growth and profuse sporulation on the outer surfaces of both leaf and root tissues beginning 8 days after inoculation. The conidia and spore-bearing phialides on symptomatic plants resembled the structures observed from pure cultures of *Neotyphodium* grown on PDA plates. Symptomatic wheat had a cottony ring of mycelia

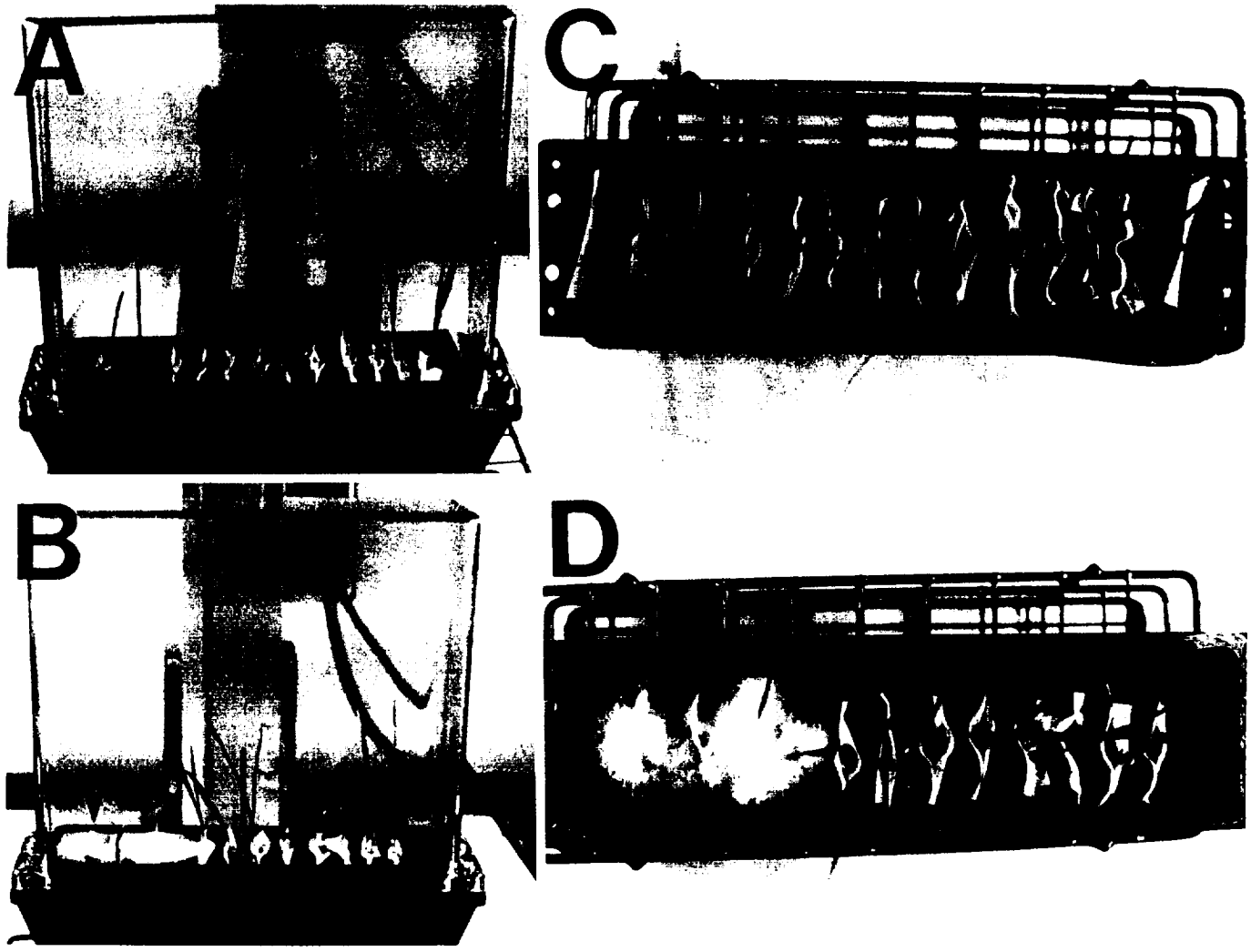

Fig. 3. Growth of wheat cv. Super Dwarf for 8 days in prototype plant growth chambers used in U.S. space shuttle missions. Chambers A and C were planted with endophyte-free, heat-treated seed from lot 2. Chambers B and D were planted with 1 *Neotyphodium*-infected seed from lot 2 (arrow) and 11 other endophyte-free seeds.

girdling the leaf sheaths, whereas leaves appeared chlorotic and twisted at growing points. Root surfaces were brown and had profuse mycelial growth. Control plants raised from noninoculated seed from lot 5 lacked these symptoms or any detectable fungal growth when grown in closed Magenta boxes or open in the greenhouse.

The extent of symptoms on wheat was related to the inoculation dose of *Neotyphodium* spores provided to the seed (Table 4). Cv. Super Dwarf showed symptoms even when inoculated with 10^2 spores per ml. Emergence of cv. Super Dwarf was less affected by *Neotyphodium* than was cv. Malcom (Table 4). Cvs. Apogee and Tam 107 were even more sensitive, with disease evident on all plants at an inoculum dose of 10^2 spores per ml (S. I. Kwon and A. J. Anderson, *unpublished data*). Inoculation of cv. Super Dwarf seed with 10^6 spores per ml resulted in clear symptom development within 8 days and, therefore, was employed in our subsequent experiments. Disease from symptomatic plants grown from naturally endophyte-infected (lot 2) seed spread to seedlings grown from endophyte-free (lot 5) seed in the enclosed PGCs housed in a prototype PGU. The plants receiving secondary inoculum showed mild to severe disease symptoms after 4 to 5 days (Fig. 3). By 8 days, conidia were found by microscopic inspection on the outer surfaces of leaf sheaths and leaves of asymptomatic plants at a distance of 7 to 18 cm from the *Neotyphodium*-infected seed source in the enclosed PGCs.

Microscopic examination of wheat leaf sheaths and root tissues of cv. Super Dwarf was used to further delineate the growth of the *Neotyphodium* fungus in asymptomatic and symptomatic seedlings. Only intercellular hyphae were present in the leaf sheaths of asymptomatic plants, which were obtained from naturally endo-

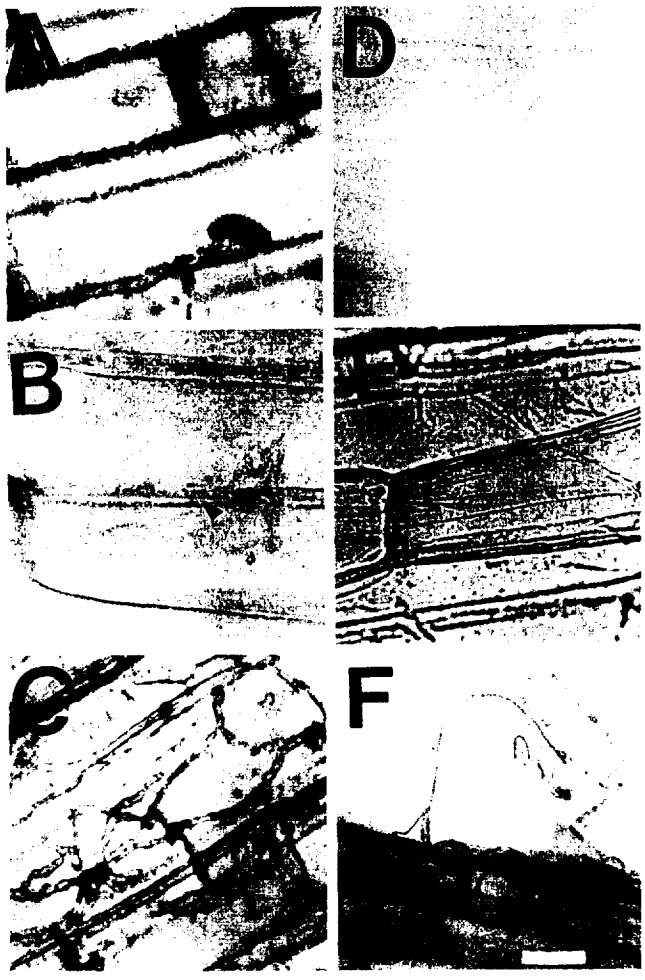

Fig. 4. Histochemical staining of endophyte-free and symptomatic 8-day-old wheat cv. Super Dwarf. Endophyte-free plants were raised in closed Magenta (Magenta Corporation, Chicago) boxes from seed (lot 5). Asymptomatic plants were generated from endophyte-infected seed (lot 2) grown under open greenhouse conditions. Symptomatic plants were from seed (lot 5) inoculated with a spore suspension of the *Neotyphodium* isolate from wheat and grown in closed Magenta boxes. Tissues were stained with aniline blue, lactic acid and photographed under 100× magnification with brightfield microscopy, as described in text. **A,** Endophyte-free leaf sheath; **B,** *Neotyphodium*-infected asymptomatic leaf sheath, the arrow shows the position of hyphae growing intercellularly through the plant cell wall; **C,** *Neotyphodium*-infected symptomatic leaf sheath; **D,** endophyte-free root; **E,** asymptomatic root; and **F,** root from *Neotyphodium*-infected symptomatic plant. The bar represents 10 μm.

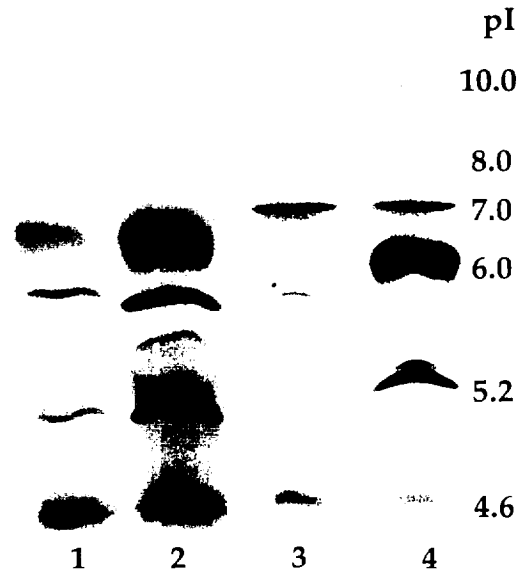

Fig. 5. Isozymic composition of total peroxidases from wheat roots (lanes 1 and 2) and leaves (lanes 3 and 4) after growth for 8 days in microgravity (lanes 2 and 4) or on earth in an orbital simulation chamber (lanes 1 and 3). Plants were grown from endophyte-infected wheat cv. Super Dwarf seed (lot 2). Total proteins were separated by isoelectric focusing gel electrophoresis and stained for peroxidase activity. The isoelectric points of the marker proteins are shown on the right.

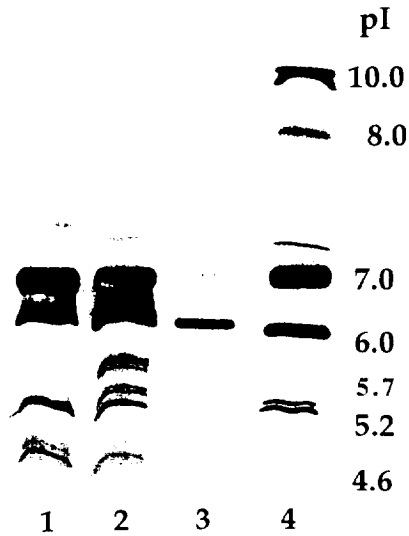

Fig. 6. Isozymic composition of total peroxidases from roots (lanes 1 and 2) and leaf sheaths (lanes 3 and 4) from 8-day-old wheat plants grown from *Neotyphodium*-inoculated (lanes 2 and 4) and noninoculated (lanes 1 and 3) seed from lot 5. Total proteins were separated by isoelectric focusing gel electrophoresis, and gels were stained for peroxidase activity. The isoelectric points of the marker proteins are shown on the right.

phyte-infected seed (lot 2) grown in the greenhouse (Fig. 4B), and no sporulation was evident. These hyphae did not appear to be convoluted, as has been observed with hyphae of other *Neotyphodium* species. No hyphae were found in the root tissues of the asymptomatic plants (Fig. 4E). In contrast, plants grown in closed containers from heat-treated, surface-sterilized (lot 2) seed inoculated with *Neotyphodium* spores displayed the visual symptoms described above, with abundant hyphae and conidia on the outer surfaces of both root and leaf tissues (Fig. 4C and F). Control plants from endophyte-free, noninoculated seed had no fungal hyphae intercellularly or on the epidermis of leaf sheaths (Fig 4A) or roots (Fig 4D).

Pathogenicity of the *Neotyphodium* isolate from wheat on other plant hosts. The *Neotyphodium* isolate from wheat caused symptoms on other grasses and cereals after spore inoculation of seed and growth under suboptimal conditions in closed containers. Germination of *D. glomerata*, *Hordeum vulgare*, *T. aestivum*, and *Zea mays* was reduced by more than 50% (Table 5). Germination of *F. arundinacea*, with or without its endophyte, *N. coenophialum*, was not reduced. *Neotyphodium* mycelia was present on the leaf surfaces and roots of *D. glomerata* and *T. aestivum* at 8 days postinoculation. *Neotyphodium* mycelia and conidia were found on the leaf surfaces of all inoculated hosts by 16 days postinoculation when assessed microscopically. Disease symptoms of chlorosis and twisting only appeared on *F. arundinacea* and *Z. mays* leaf sheaths and leaves at 16 days postinoculation, when *Neotyphodium* mycelia also were visible on the vermiculite surface. The leaves from all inoculated plants were shorter in length. *F. arundinacea* infected with *N. coenophialum* showed the least extent of infection of leaf sheaths. Roots of *Festuca*, *Zea*, or *Hordeum* were not infected by 16 days postinoculation, although progressive colonization was detected in *Dactylis*.

Plant peroxidases and *Neotyphodium* colonization. The goal of the space mission was to examine whether there are changes in peroxidase activity in wheat after growth in microgravity. Thus, extracts were prepared from both the spaceflight- and earth-grown plants with seedlings that were visually free of disease symptoms. Different isozymes of peroxidase were detected in extracts of roots (pI 5.7) and leaf sheaths (pI 5.2) from the microgravity-grown wheat seedlings compared to extracts from the control seedlings grown simultaneously on earth (Fig. 5). However, the unique isozymes in extracts from the microgravity-grown plants also were found in symptomatic plants derived from the microbial-free lot 5 seed inoculated with the *Neotyphodium* isolate from wheat and grown in closed containers (Fig. 6). These induced isoforms of peroxidase were not detected in extracts from seedlings from asymptomatic noninoculated lot 5 seed.

DISCUSSION

Microbiological surveys of spaceflight missions have found microbes that have the potential to colonize plants (4,26,30,31). Some of these organisms are likely to be potential plant pathogens, and their pathogenicity may be enhanced by spaceflight conditions. The wheat cv. Super Dwarf seed allocated to the spaceflight mission flown on the U.S. space shuttle Discovery during February 1995 contained a fungal endophyte with the ability to produce symptoms on spaceflight and ground-control plants during growth under closed suboptimal conditions. The symptoms were caused by a seedborne *Neotyphodium* species, and thus, the fungus was propagated via maternal transmission. The wheat endophytic fungus was identified as a *Neotyphodium* species based on its production of smooth hyaline conidia from characteristic phialides (23,24,34,36). The conidial size, 10 to 20 µm long and 1.0 to 2.0 µm wide, falls within the size range reported for other species of *Neotyphodium*. For example, *N. chilense* conidia are between 10 and 30 µm long and 1 and 1.5 µm wide, whereas those from a *Neotyphodium* species associated with *Stipa robusta* are between 5.9 and 9.7 µm long and 2.0 and 3.4 µm wide (15,23,24,34,36).

Two protein bands from the wheat fungal isolate separated by SDS-PAGE cross-reacted with antibodies generated to *N. coeno-*

TABLE 5. Symptoms caused by inoculation of grass and cereal seed with the *Neotyphodium* isolate from wheat at 8 and 16 days

		Symptoms[z]			
		Leaf sheaths (%)		Roots (%)	
Host species[y]	Germination (%)	8 days	16 days	8 days	16 days
Dactylis glomerata					
Control	100 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
+*Neotyphodium*	37 ± 6	59 ± 13 b	100 ± 0 a	24 ± 6 b	24 ± 6 b
Festuca arundinacea					
KY31, endophyte positive					
Control	100 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
+*Neotyphodium*	100 ± 0	0 ± 0 d	17 ± 4 d	0 ± 0 c	0 ± 0 c
KY31, endophyte negative					
Control	100 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
+*Neotyphodium*	100 ± 0	0 ± 0 d	43 ± 10 c	0 ± 0 c	0 ± 0 c
Hordeum vulgare					
Control	95 ± 5	0 ± 0	0 ± 0	0 ± 0	0 ± 0
+*Neotyphodium*	25 ± 6	100 ± 0 a	100 ± 0 a	0 ± 0 c	0 ± 0 c
Triticum aestivum					
Cv. Super Dwarf					
Control	82 ± 18	0 ± 0	0 ± 0	0 ± 0	0 ± 0
+*Neotyphodium*	21 ± 5	100 ± 0 a	100 ± 0 a	100 ± 0 a	100 ± 0 a
Zea mays					
Control	63 ± 14	0 ± 0	0 ± 0	0 ± 0	0 ± 0
+*Neotyphodium*	10 ± 3	0 ± 0 d	100 ± 0 a	0 ± 0 c	0 ± 0 c

[y] Seeds of the plants listed were inoculated with a suspension of *Neotyphodium* spores, as described in text. Noninoculated control plants received only surface-sterilization treatments. The plants were grown in closed Magenta boxes (Magenta Corporation, Chicago) and examined for symptoms at 8 and 16 days post-planting.

[z] Percentage of plants with symptoms on leaf sheaths and roots was estimated for each plant host. Data were transformed by arcsine square-root transformation and are represented as back-transformed means with standard error of five replications each with 20 plants. Comparisons between host species were made for the *Neotyphodium*-inoculated plants. Differences were found between host species in percent infection of roots and leaves at both 8 and 16 days after inoculation with the *Neotyphodium* wheat isolate ($\alpha \leq 0.05$). Means in the same column followed by the same letter are not significantly different.

phialum, an endophyte of fescue. Thus, our results agree with the findings of C. West, University of Arkansas, Fayetteville (*personal communication*), that these polyclonal antibodies raised against *N. coenophialum* recognize other *Neotyphodium* species. Kaiser et al. (15) also reported on an antibody preparation that recognizes different *Neotyphodium* isolates. However, the aggressive growth habit of the wheat isolate on carbohydrates, especially xylose (24), and its profuse sporulation and ability to cause disease on wheat under stressed conditions differ from that of *N. coenophialum* and other related *Neotyphodium* species classified in section *Albo-lanosa*. Generally, these fungal endophytes grow slowly in culture, have limited spore production, and are asymptomatic on their hosts. Some species of *N. typhinum* have been classified as type I, II, or III according to their pathogenic potential (36). Type III isolates cause symptoms of "choke," which are characterized by the formation of fungal reproductive structures or stromata, which replace the inflorescences (36).

Neotyphodium species in section *Albo-lanosa* are found predominately in the sheath and are not found in roots (23,24,36). However, Kaiser et al. (15) reported that mycelia were detected when roots of *Stipa robusta* infected with an uncharacterized *Neotyphodium* species were plated on PDA medium. They also observed mycelia erupting from the leaf tips of endophyte-positive *S. robusta*, a phenomenon that we also have observed in symptomatic wheat. Another *Neotyphodium* species, *N. chilense*, is more aggressive in its interaction with its host and grows either intercellularly or intracellularly in orchard grass (24). Morgan-Jones et al. (24) suggested that this *Neotyphodium* endophyte may become pathogenic under conditions that promote plant stress or during tissue senescence. The *Neotyphodium* isolate from wheat resembles *N. chilense* in its spore and phialide morphology, as well as in its ability to produce purple pigmentation when grown on a medium of wheat meal or isolated wheat cell walls (A. J. Anderson, *unpublished data*). It is unknown whether *Neotyphodium*-infected wheat produces alkaloids or other metabolites that may produce toxins to deter insects. Preliminary results showed that, in contrast to reports for *N. coenophialum* (35), metabolites produced by the fungus after growth on PDA did not inhibit the growth of other fungal plant pathogens (A. J. Anderson and B. Issa, *unpublished data*).

The *Neotyphodium* isolate from wheat was asymptomatic in wheat grown under normal greenhouse cultivation, e.g., no visible mycelial growth on plant tissues was apparent and flowering and seed set were unaffected. Asymptomatic growth of *Neotyphodium* in Super Dwarf wheat occurred intercellularly in leaf sheaths, with no visible signs of infection in or on the root tissues. This interaction between wheat and *Neotyphodium* resembled the asymptomatic growth of other *Neotyphodium* isolates in grasses (6,28, 29,36). In contrast, wheat grown under closed conditions, similar to those in spaceflight from *Neotyphodium*-infected seed, displayed profuse external growth of mycelia and extensive sporulation. Other symptoms of *Neotyphodium* infection included mycelial girdling of the leaf sheath accompanied by chlorosis and narrowed or twisted leaf tissues. Plant growth under spaceflight conditions apparently provided a conducive environment for this *Neotyphodium* species to proliferate and cause disease. Identical disease symptoms also were observed on other plant hosts grown in closed containers after inoculation of seed with the *Neotyphodium* wheat isolate. Thus, the fungus is not restricted in its host range under these growth conditions. Whereas various species of *Neotyphodium* commonly are reported as endophytes in asymptomatic grasses (6), such as *Festuca* (8,23), *Dactylis* (24), and *Stipa* (15), to our knowledge the effects of *Neotyphodium* applied artificially as a spore suspension in a grass-endophyte interaction never have been assessed. It has been proposed that the *Neotyphodium* endophytes arose from *Epichloe* species, which may be pathogenic and result in disease or "choke" of seed-bearing heads (21,29), and other *Neotyphodium* isolates have been documented as pathogens on other nongrass hosts. For example, *Acremonium* species have been documented as the causal agents of muskmelon collapse (9). In this particular case, the fungus resulted in symptoms predominately in the roots, with little effect on leaves until late in disease development.

For meaningful plant growth and development experiments to be carried out in space, the effects of microbial interactions must be considered. Our findings support the need to screen germ plasm used in spaceflight research to detect surface and endophytic microbial contamination. For example, the introduction of just one *Neotyphodium*-infected seed can result in infection of and deleterious consequences to other plants in the same closed growth module. In addition, we found that the peroxidase isozyme pattern of wheat cv. Super Dwarf was altered by the presence of the *Neotyphodium* endophyte (3), making it impossible to fulfill our original goal of identifying the effects of microgravity on plant peroxidase composition. We recommend that plants be examined routinely for microbial sterility at the end of spaceflight missions to ensure that any changes are in response to microgravity and not due to the complications of microbial contamination. Strategies to control pathogens in space are essential to the development of adequate plant growth conditions for future plant experiments on the International Space Station and to maintain the quality and production of food resources in space.

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